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Synthesis of Peptides Related to Substance P and Their Activities as Agonists and Antagonists

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Seven analogs of substance P (SP) were synthesized by a solid-phase technique. Tests for agonistic and antagonistic activities were performed on the isolated guinea pig ileum. [D-Phe^9]-SP had very low agonistic activity, but no significant antagonistic activity. [Ile^7, Ile^8]-SP had low activity but [Ile^8]-SP was almost twice as active as SP. Heptapeptide analogs of [Ile^8]-SP having a basic L or D-amino acid at the *N*-terminal (Lys or D-Arg) were almost as active as SP.

The availability of the synthetic substance P (SP), Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2, after its isolation and identification [1,2] led to increasing interest in its pharmacological properties, and the relative importance of the eleven amino acids for binding and activation of mechanisms. In a previous report [3] we cited the background and physiological significance of SP, and a group of related naturally occuring peptides, generally summarized as tachikinins. Before the structure of SP was known, its similarity to physalemin and eledoisin was recognized.[4] A structural similarity between these peptides, particularly at the *C*-terminal, was revealed after the sequence of SP was elucidated. Consequently,

* PH. 136.

Table 1. Data on agonist and antagonist activities.

No.	Substance	Potency relative to SP on the isolated guinea pig ileum	Antagonist activity [a]
SP	Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2 (Substance P)	100	
1.	Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-D-Phe-Leu-Met-NH_2 ([D-Phe^9]-SP)	0.5	−
2.	Arg-Pro-Lys-Pro-Gln-Gln-Phe-Ile-Gly-Leu-Met-NH_2 ([Ile^8]-SP)	180	+
3.	Arg-Pro-Lys-Pro-Gln-Gln-Ile-Ile-Gly-Leu-Met-NH_2 ([Ile^7, Ile^8]-SP)	0.05	
4.	Arg-Pro-D-Leu-Pro-Gln-Gln-Ile-Phe-D-Phe-Leu-Met-NH_2 ([D-Leu^3, Ile^7, D-Phe^9]-SP)	0.02	−
5.	Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-NH_2 ([des-Gly^9, des-Leu^{10}, des-Met^{11}]-SP)	< 0.01	−
6.	Lys-Gln-Phe-Ile-Gly-Leu-Met-NH_2	30	−
7.	D-Arg-Gln-Phe-Ile-Gly-Leu-Met-NH_2	110	−

[a] Concentration, 10^6 M. −, negative; +, positive.

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the early structure-activity studies on eledoisin,[5] can be useful for the design of new SP analogs.

A prominent feature in structure-activity relationships of SP, is the fact that the *C*-terminal pentapeptide exhibits low activity, but sequences of six or more *C*-terminal amino acids are necessary for activity comparable to that of SP.[6] This knowledge opened the possibility for the synthesis of highly active shortened SP analogs.

The assumption has been made that the pentapeptide-sequence is necessary for "basic biological information", and that the *N*-terminal moiety provides a "reinforcement effect".[7] In other words, the *C*-terminal pentapeptide appeared essential for "intrinsic efficacy" with the *N*-terminal sequence participating in the binding to a receptor.

It was found that Phe-7 of SP is significant for activity, but Phe-8 is not. When Gly-9 was replaced by D-Leu, the activity decreased. [D-Phe^7]-SP had weak antagonistic activity.[3]

Continuing this research, we now describe the synthesis of seven new analogs of SP. Tests for agonist and antagonist activity were performed on the isolated guinea pig ileum, and the results are given in Table 1.

BIOLOGICAL METHODS

The agonist activities of the synthetic analogs and of substance P were compared using the terminal portion of the guinea pig ileum. Concentration-response curves were obtained by adding the analog cumulatively so that the concentration in the bath was increased by a factor of 2 whenever a steady response to the previous concentration had been reached. In the tests for antagonistic activity, the analog of substance P was added 10 min before substance P was added.[8]

SYNTHETIC METHODS AND PURIFICATION

Amino acids and the BHA resin were purchased from Beckman Inc., Palo Alto, Calif., or from Bachem Inc., Marina del Rey, Calif.

For amino acid analysis, samples were hydrolyzed in 6 M HCl in evacuated sealed glass ampoules at 110 °C for 18 h. The hydrolysate was evaporated and was dissolved in a sodium citrate buffer (pH 2.2). This solution was injected into a Beckman Model 119 Amino Acid Analyzer equipped with an Infotronics Model CRS-210 digital integrator. After the first amino acid is coupled to the BHA resin, an analysis for the amount of the amino acid was not required, because the ninhydrin test revealed complete coupling. The BHA resin as purchased was specified to contain 0.47 meq. N/g. A complete coupling of the amino acid derivative, as proved by the ninhydrin color test, is reached in every case.

Homogeneity of the peptides was demonstrated by thin layer chromatography on silica gel plates, using the following solvent systems: R_{F1}, n-BuOH – EtOAc – AcOH – H_2O, (1:1:1:1); R_{F2}, EtOAc – pyridine – AcOH – H_2O, (5:5:1:3); R_{F3}, n-BuOH – pyridine – AcOH – H_2O, (30:30:6:24); R_{F4}, i-Prop-1 N AcOH, (2:1); R_{F5}, $CHCl_3$ – conc. NH_4OH – CH_3OH, (60:20:45); R_{F6}, n-BuOH – EtOAc – AcOH – H_2O, (2:2:1:1).

Each purified product showed only one spot in all of the six systems used as detected with the ninhydrin and chlorine – *o*-tolidine reagents. The analogs were synthesized by the solid phase method [9,10] and couplings were performed with a Beckman Model 990 Peptide Synthesizer. The benzhydrylamine (BHA) resin [11] was used as a solid support. The α-amino functions were protected by Boc groups except for D-Arg and <Glu where the Z group was used, and Arg where Aoc was used.

The side chain functionalities were protected by Tos (Arg) and 2-Cl-Z (Lys). The first Boc amino acid was coupled to the BHA resin by the DCC method. Subsequent Boc-amino acids were attached to the solid support by the DCC or by the active ester (Gln) coupling program as described.[3]

1. [D-Phe^9]-SP (Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-D-Phe-Leu-Met-NH_2). Starting from 5 g BHA resin (HCl salt), 5.5 g Leu-Met-BHA resin were prepared. To 2.5 g of this peptide resin, the appropriate Boc-amino acid derivatives were coupled to yield 3.6 g Boc-Arg(Tos)-Pro-Lys(2-Cl-Z)-Pro-Gln-Gln-Phe-Phe-D-Phe-Leu-Met-BHA resin. Cleavage by HF gave 900 mg of the crude undecapeptide. Purification by gelfiltration chromatography on Bio-Gel P2 with 1.3 % acetic acid yielded a partial purified peptide. Further purification was performed by partition chromatography on Sephadex G-25 with 0.1 % AcOH – n-BuOH – pyridine (10:7:3), and 60 mg of the pure peptide were obtained. Amino acid analysis: 2 Glu 1.20 × 2, 2 Pro 1.09 × 2, Met 0.86, Leu 0.85, 3 Phe 1.15 × 3, Lys 0.88, Arg 0.90. R_F Values: $R_{F1} = 0.53$; $R_{F2} = 0.73$; $R_{F3} = 0.61$; $R_{F4} = 0.04$; $R_{F5} = 0.28$.

2. [Ile ^{8}]-SP (Arg-Pro-Lys-Pro-Gln-Gln-Phe-Ile-Gly-Leu-Met-NH_2). Starting with 3 g Boc-Gly-Leu-Met-BHA resin, 4.5 g Aoc-Arg(Tos)-Pro-Lys(2-Cl-Z)-Pro-Gln-Gln-Phe-Ile-Gly-Leu-Met-BHA resin was synthesized. Cleavage by HF yielded 1.2 g of the crude peptide. An aliquot of 100 mg was purified first by gel filtration chromatography on Bio-Gel P2 with 1.3 % AcOH. Another purification by partition

chromatography on Sephadex G-25 with 0.1 %. AcOH – n-BuOH – pyridine (10:7:3) was necessary to obtain 18 mg pure peptide. Amino acid analysis: 2 Glu 1.20 × 2, 2 Pro 1.10 × 2, Gly 0.82, Met 0.91, Ile 0.79, Leu 0.93. Phe 0.97, Lys 0.94, Arg 0.99. R_F Values: $R_{F1} = 0.19$; $R_{F2} = 0.68$; $R_{F3} = 0.56$; $R_{F4} = 0.04$; $R_{F5} = 0.10$.

3. [Ile ^{7}, Ile ^{8},]-SP (Arg-Pro-Lys-Pro-Gln-Gln-Ile-Ile-Gly-Leu-Met-NH_2). From two grams of BHA resin (HCl salt), 3.37 g of Aoc-Arg(Tos)-Pro-Lys (2-Cl-Z)-Pro-Gln-Gln-Ile-Ile-Gly-Leu-Met-BHA resin were synthesized. Cleavage by HF yielded 1.0 g of crude peptide. A portion of 210 mg was first purified by partition chromatography on LH-20 with n-BuOH-EtOAc-pyridine-0.1 % AcOH (7:2:1:10). The main fraction (182 mg) was further purified by partition chromatography on Sephadex G-25 with 0.1 % AcOH-n-BuOH-pyridine (11:5:3), and 16 mg of pure peptide were obtained. Amino acid analysis: 2 Glu 1.05 × 2, 2 Pro 1.15 × 2, Gly 0.96, Met 0.90, 2 Ile 0.96 × 2, Leu 0.94, Lys 0.91, Arg 0.94. R_F = Values: $R_{F1} = 0.05$; $R_{F2} = 0.46$; $R_{F3} = 0.44$; $R_{F1} = 0.02$; $R_{F5} = 0.08$.

4. [D-Leu ^{3}, Ile ^{7}, D-Phe ^{9}]-SP (Arg-Pro-D-Leu-Pro-Gln-Gln-Ile-Phe-D-Phe-Leu-Met-NH_2). Starting with 2.5 g BHA resin (HCl salt), 3.83 g of Aoc-Arg(Tos)-Pro-D-Leu-Pro-Gln-Gln-Ile-Phe-D-Phe-Leu-Met-BHA resin were synthesized. Cleavage by HF yielded 1.2 g of the crude peptide. An aliquot of 200 mg was purified by partition chromatography on Sephadex G-25 with n-BuOH-EtOAc-AcOH-H_2O (2:2:1:1) and 86 mg partially purified peptide was obtained. Another partition chromatography of this product on Sephadex LH-20 with n-BuOH-EtOAc-pyridine-0.1 % AcOH (7:2:1:10) yielded 40 mg of the pure peptide. Amino acid analysis: 2 Glu 1.09 × 2, 2 Pro 1.07 × 2, Met 0.85, Ile 0.86, 2 Leu 1.05 × 2, 2 Phe 1.03 × 2, Arg 0.87. R_F Values: $R_{F1} = 0.56$; $R_{F2} = 0.76$; $R_{F3} = 0.62$; $R_{F4} = 0.43$; $R_{F5} = 0.56$; $R_{F6} = 0.35$.

5. [des-Gly ^{9}, des-Leu ^{10}, des-Met ^{11}]-SP (Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-NH_2). From 2.4 g of BHA resin (HCl salt), 2.95 g of Aoc-Arg-(Tos)-Pro-Lys-Pro-Gln-Gln-Phe-Phe-BHA resin were synthesized. Cleavage by HF gave 320 mg of the crude peptide which were first purified by partition chromatography on Sephadex G-25 eluted with 0.1 % AcOH-n-BuOH-pyridine (10:7:3). The peptide was retained on the column when eluted with the upper phase, but was eluted with the lower phase, and 175 mg of partially purified peptide were obtained. This fraction was further purified by partition chromatography on Sephadex LH-20 with n-BuOH – EtOAc – Pyridine – 0.1 % AcOH (7:2:1:10), and 128 mg of the purified peptide were recovered. Further purification of this fraction on Sephadex G-25 with 0.1 % AcOH – n-BuOH – pyridine (11:5:3) gave 34 mg of the pure peptide. Amino acid analysis: 2 Glu 1.13 × 2, 2 Pro 0.91 × 2, 2 Phe 1.04 × 2, Lys 0.85, Arg. 0.98. R_F Values: $R_{F1} = 0.05$; $R_{F2} = R_{F3} = 0.39$; $R_{F4} = 0.02$; $R_{F5} = 0.04$.

6. Lys-Gln-Phe-Ile-Gly-Leu-Met-NH_2. Boc-Lys(2-Cl-Z) was coupled to 1 g of Boc-Gln-Phe-Ile-Gly-Leu-Met-BHA resin. The resulting heptapeptide resin was cleaved by HF and yielded 200 mg crude peptide. Purification was performed by gelfiltration on Bio-Gel P-2 with 1.3 % acetic acid. The main fractions were further purified by partition chromatography on G-25 with 0.1 % AcOH – n-BuOH – pyridine (10:7:3) to yield 20 mg pure peptide. Amino acid analysis: Glu 1.09, Gly 1.0, Met 0.91, Ile 0.94, Leu 1.06, Phe 1.02, Lys 0.99. R_F Values: $R_{F1} = 0.60$; $R_{F2} = 0.76$; $R_{F3} = 0.69$; $R_{F4} = 0.54$; $R_{F5} = 0.71$.

7. D-Arg-Gln-Phe-Ile-Gly-Leu-Met-NH_2. Starting from 1 g of Boc-Gln-Phe-Ileu-Gly-Leu-Met-BHA resin, 1.2 g Z-Arg(Tos)-Gln-Phe-Ile-Gly-Leu-Met-BHA resin was obtained. After cleavage by HF, 250 mg of the crude peptide were first purified by gelfiltration on Bio-Gel P-2 with 1.3 % acetic acid. Further purification by partition chromatography on Sephadex G-25 with 1.0 % AcOH – n-BuOH – pyridine (10:7:3) yielded 30 mg pure heptapeptide. Amino acid analysis: Glu 1.13, Gly 1.02, Met 0.90, Ile 1.08, Phe 1.00, Arg 0.97. R_F Values: $R_{F1} = 0.63$; $R_{F2} = 0.79$; $R_{F3} = 0.73$; $R_{F4} = 0.55$; $R_{F5} = 0.33$.

RESULTS AND DISCUSSION

It was shown [3] that substitution of Gly in position 9 by D-Leu leads to a great loss of activity (0.5 %) in comparison with SP. Substitution of Gly in position 9 by D-Phe to give the analog *1* resulted in the same loss in activity as by substitution with D-Leu.

Recent NMR studies [12] on SP revealed the possibility of a hydrogen bond between Met-11 and Gln-5. Inspection of an atomic model of SP having this hydrogen bond, indicates that the *C*-terminal hexapeptide forms a turn, in which the α protons in the L-configuration are directed to the inside of this turn. If one of the five *C*-terminal amino acids has a D-configuration, the side-chains are directed to the inside of the turn and steric hindrance could inhibit this hydrogen bonding. Especially, Gly-9 with its rotational freedom seems to be important for this conformation.

Eledoisin and phyllomedusin have Ile in the position 5, counting from the *C*-terminal.[13] So, it is not surprising that [Ile ^{8}]-SP (*2*) has higher activity than SP (180 %). [Ile ^{7}, Ile ^{8}]-SP had only 0.05 % of the activity of SP and shows the necessity of Phe in position 7. [Ile ^{8}]-SP had weak antagonistic activity, but [Ile ^{7}, Ile ^{8}]-SP (*3*) did not have antagonistic activity under comparable conditions.

In analog *4*, Phe in position 7 was changed to Ile and Gly-9 to D-Phe to obtain expected low activity (0.02 %). Additional substitution of Lys-3 by D-Leu ^{3} was made in analog *4*. Although *4* had low activity, there was no antagonistic activity.

Compounds 6 and 7 represent two analogs of a group of highly active SP analogs. Heptapeptide analogs of SP are active when Phe-8 of SP is exchanged by Ile, and the *N*-terminal is a basic L- or D-amino acid (Lys or D-Arg).

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